Advanced-Stage Cervical Carcinomas Are Defined by a Recurrent Pattern of Chromosomal Aberrations Revealing High Genetic Instability and a Consistent Gain of Chromosome Arm 3q

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We have analyzed 30 cases of advanced-stage cervical squamous cell carcinoma (stages IIb–IV) by comparative genomic hybridization (CGH). The most consistent chromosomal gain in the aneuploid tumors was mapped to chromosome arm 3q in 77% of the cases. Acquisition of genetic material also occurred frequently on 1q (47%), 5p (30%), 6p (27%), and 20 (23%). Recurrent losses were mapped on 2q (33%), 3p (50%), 4 (33%), 8p (23%), and 13q (27%). High-level copy number increases were mapped to chromosome 8, chromosome arms 3q, 5p, 8q, 12p, 14q, 17q, 19q, 20p, and 20q, and chromosomal bands 3q26-27, 9p23-24, 11q22-23, and 12p13. In the majority of the cases, the presence of high-risk human papilloma virus genomes was detected. High proliferative activity was accompanied by crude aneuploidy. Increased p21/WAF-1 activity, but low or undetectable expression of TP53 were representative for the immunophenotype. This study confirms the importance of a gain of chromosome arm 3q in cervical carcinogenesis and identifies additional, recurrent chromosomal aberrations that are required for progression from stage I tumors to advanced-stage carcinomas. *Genes Chromosom. Cancer 19:233–240, 1997.* © **1997 Wiley-Liss, Inc.**[†]

INTRODUCTION

Cervical carcinomas are the second most common tumors in women worldwide. The tumor incidence shows strong variability, with industrialized countries having lower morbidity and mortality rates than do developing countries (Pisani et al., 1993). This variation is attributable mostly to cytologic screening programs (Hakama et al., 1986). Although other factors, such as cigarette smoking, may influence the incidence of cervical cancer, infection with human papilloma virus (HPV) is the initiating event for cervical carcinogenesis (IARC, 1995). It is conceivable, however, that additional, genetic aberrations are required for the multistep process of tumor initiation and progression (Fearon and Vogelstein, 1990; zur Hausen, 1994). Determination of mandatory genetic aberrations for tumor progression would potentially lead to diagnostic predictors. The search for recurrent chromosomal aberrations in cervical carcinomas, however, was hampered by technical difficulties in karyotype analysis by means of chromosome banding techniques (Atkin et al., 1990). We have therefore used comparative genomic hybridization (CGH) to identify recurrent gains and losses in cervical carcino-

mas. CGH serves as a molecular cytogenetic screening test that allows mapping of chromosomal copy number changes on normal metaphase chromosomes (Kallioniemi et al., 1992). Therefore, tumor cell culture is not required, and archival tissue sections that were formalin fixed and paraffin embedded can be used for analysis (Speicher et al., 1993; Isola et al., 1994). This feature of CGH is particularly useful when a phenotype/genotype analysis in solid tumor progression is attempted (Ried et al., 1996). We have recently applied CGH to identify chromosomal aberrations that occur during the genesis of cervical carcinomas. The analysis of DNA extracted from normal cervical epithelium, mild, moderate, and severe dysplasias, and invasive carcinomas (stage I) has shown that the emergence of copy number increases of chromosome arm 3q defines the transition from preinva-

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Received 10 October 1996; Accepted 7 January 1997

sive to invasive tumors (Heselmeyer et al., 1996). In order to explore the relevance of the gain of chromosome arm 3q in advanced stages of tumor progression and to identify additional copy number changes, we have performed CGH on 30 cases of stage IIb to IV squamous cell carcinomas of the uterine cervix.

MATERIALS AND METHODS

Tissue Samples

Thirty tumors were collected at the Karolinska Hospital, Stockholm, Sweden, between 1991 and 1995. All tumors were diagnosed as cervical squamous cell carcinomas. Patient age and tumor staging are presented in Table 1. The tumors were staged based on clinical and histologic assessment according to the International Federation of Gynaecology and Obstetrics (FIGO) (Shepherd, 1989). Consecutive sections were cut from all formalin-fixed, paraffin-embedded samples. The tissue sections were used for DNA ploidy measurements (thickness 8 μm), immunohistochemistry (4 μm), and tumor DNA extraction (50 μm). The last section (4 μm) was stained with hematoxylin-eosin (H&E) for confirmation of the presence of tumor tissue. All tumor samples were obtained before treatment.

DNA Cytometry

DNA content measurements were performed by image cytometry on Feulgen-stained histologic sections as described (Auer et al., 1989; Auer et al., 1994). All DNA values were expressed in relation to the corresponding staining controls, which were given the value 2c, indicating the normal diploid DNA content. Three classes of histograms were obtained: 1) tetraploid cases with a main peak in the 4c region and no cells or only a minor fraction of cells (less than 5%) exceeding 5c; 2) aneuploid/proliferating tetraploid cases with a peak in the 4c region and a fraction of 5–20% of the cells exceeding 5c; and 3) aneuploid cases with a main peak around the 4c region and more than 20% of the cells exceeding 5c. Examples of the histograms are shown in Figure 1.

Immunohistochemistry

Proliferative activity was determined with an antibody (MIB-1) directed against the Ki-67 antigen. The antibody distinguishes cells in G0 from cells that are in G1-S-G2-M phase of the cell cycle. p21/WAF-1 expression was analyzed with the WAF-1 antibody (Oncogene Sciences, Uniondale, NY), and TP53 expression was determined with the DO-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunohistochemistry was performed essentially as described (Steinbeck et al., 1995). The quantification of the results is presented in Table 1.

HPV Genotyping

HPV genomes in the purified DNA samples were identified by polymerase chain reaction (PCR), with the MY09-MY11 L1 consensus primers used for amplification (Bosch et al., 1995). We used 25 type-specific probes and one generic HPV probe to diagnose the HPV type in the PCR products (Hildesheim et al., 1994). β-globin amplification was used as a positive control for evaluation of the adequacy of tumor DNA.

CGH

CGH was performed according to standard procedures (Heselmeyer et al., 1996). Tumor DNA was extracted from sodium isothiocyanate-treated, formalin-fixed tissue sections as described (Ried et al., 1995). Nick translation was performed with bio-16-dUTP (tumor DNA) and digoxigenin-11-dUTP (reference DNA, prepared from a karyotypically normal female donor). Five hundred nanograms of each of the labeled genomes was combined and hybridized with an excess of Cot-1 DNA (25 μg) to metaphase chromosomes prepared from a karyotypically normal donor. The biotin-labeled tumor genome was visualized with avidin conjugated to fluorescein isothiocyanate (FITC) (Vector Laboratories, Burlingame, CA), and the digoxigenin-labeled reference DNA with a mouse anti-digoxin antibody (Sigma, St. Louis, MO), followed by detection with a goat anti-mouse antibody conjugated to tetramethylrhodamine isothiocyanate (TRITC) (Sigma). Chromosomes were counterstained with DAPI to generate a G-like banding pattern.

Gray scale images of the DAPI counterstain, the FITC-labeled tumor DNA, and the TRITC-labeled reference DNA from at least 8 metaphases from each hybridization were acquired with a cooled CCD camera (CH250, Photometrics, Tucson, AZ) connected to a Leica DMRBE microscope equipped with fluorochrome-specific optical filters TR1, TR2, and TR3 (Chroma Technology, Brattleboro, VT). Quantitative evaluation of the hybridization was achieved with a custom-designed computer pro-

TABLE 1. Summary of the Clinical Data and Results From MIB-1, DO-1, WAF-1 Staining, DNA Ploidy Measurements, HPV Genotyping, and CGH in Advanced Cervical Carcinomas[a]

Case	List no.	Patient age (years)	Tumor stage	% MIB-1 (Ki-67)	% DO-1 (p53)	% WAF-1/ p21	DNA	HPV	CGH
1	C1	69	IIIb	100	Neg.	<2	6.6c, A	45	−2q36-ter,−3p21-12,+3q26.1-ter,++5p,−5q35-ter, −6q,−8pter-22,++8q, −11q,−13q11–14,−X
2	C3	84	IIIb	40	20	50	2.8c, A	16	+1q,+3(++3q26–27),−5pter-14,+7,++8,−9pter-21, −14q31-ter,−X
3	C6	46	IIb	90	Neg.	60	3.6c, A/pT	16	−2q34-ter,+3q25-ter,−6q25-ter,++9pter-23, −11q22-ter,+Xpter-q13
4	C7	50	IIIb	90	20	40	5.1c, A	39	+1q,+2p,+3q21-ter,−4p15-qter,+5p,+5q31-ter, +6p,+9q,+12p,+12q23-ter, +13q31-ter, −14q22–24,+15q24–25,+16,+17p,++17q, +19q,+20p,++20q,+22
5	C8	36	IIb	90	<2	10	8.0c, A/pT	45	+1q,−2q36-ter,+3q,−4pter-q23,−4q34-ter,+5p, −5q14–23,+6p,++11q22–23, +16,−22,+Xp
6	C9	87	IIIb	80	10	40	5.8c, A	31	+1,−2q36-ter,−10q26-ter,+12,+14q24-ter, +15,−18q23-ter,+Xq
7	C11	72	IIIb	90	10	30	4.1c, A/pT	16	+1q,+3q,++12p13
8	C13	42	IIIb	80	<2	30	6.1c, A	45	−8pter-23
9	C15	74	IIIb	70	<2	30	4.3c, A/pT	16	+1q,−3p,+3q,−8p,+15
10	C16	71	IIIb	90	Neg.	10	3.8c, A	33	−3pter-q21,+3q22-ter,+5q,−8p,+8q,−11q14-ter, −14,−16q,++20p,−X
11	C18	67	IIb	60	60	60	5.2c, A	Neg.	+6p,+6q21–22,++19q,+20p,++20q,+Xpter-q13
12	C19	62	IIb	50	20	50	3.8c, T	Neg.	+3q
13	C20	38	IIb	90	Neg.	Neg.	4.0c, A/pT	45	−3p,−4,++5p,−5q11–23,+6pter-21,−9p,+12q, +13q32-ter,−14,−17p,+17q, +19q13.2-ter,−X
14	C21	36	IIIb	50	Neg.	70	4.0c, T	16	−3p,+3q,−4,−10,−11pter-15,−11q22-ter
15	C22	74	IIb	80	<2	60	4.0c, T	16	+1,−3p,+3q,++5p,+6p,−6q,++9pter-24,−13,+X
16	C23	58	IIb	50	20	50	3.6c, T	16	+1q,−2q36-ter,−3p,+3q(++3q24-ter),−6q, −7q21-ter,+9,−10q
17	C24	46	IV	90	Neg.	60	4.2c, A/pT	16	−2pter-24,−2q32-ter,−3p21-11,+3q,−4,−13,+14
18	C26	75	IIb	60	10	40	3.8c, A/pT	Neg.	+1q,−6,−14,−Xq21-ter
19	C27	87	IIIb	60	Neg.	70	4.0c, A/pT	Neg.	+1,+2p,−3p,+3q,−5p,−9p,+X
20	C29	72	IVa	20	80	60	4.1c, A/pT	Neg.	+1q,−2q22-ter,−3p,+3q24-ter,−4q,−8p,+8q, −17p,−18q,−21
21	C30	81	IIIb	90	Neg.	40	3.8c, A/pT	31	+1q,−2q36-ter,−3p21-11,+3q23-ter,−4q,−6p, −6q26-ter,−9p,−10p, −11pter-14,+11p13-q13, −11q14-ter,−13,−17pter-12,−18,+Xq22–24
22	C31	63	IVa	80	Neg.	80	5.0c, A	52	++3q21-ter,−4p,++5pter-13,+6,−8p,+8q, +9,++12p,+12q,−13q14–22,+18,+20, +22
23	C32	79	IIIb	90	10	20	n.d.	18	+1,+3q,+6,+8,+9,+10,++14,+15,+16,+17q,−18q, +19,+20,+21
24	C33	64	IIb	80	Neg.	50	4.1c, A/pT	45	−3p,+3q,+4q21–22,−6,−13,+15q21–25
25	C34	51	IIIb	80	5	30	7.0c, A/pT	16	+5p,+9q,−12p,+12q,−X
26	C36	54	IIIb	90	5	70	6.7c, A/pT	16	+1q,+3q21-ter,+6pter-12,+9p,+17
27	C37	84	IIb	80	Neg.	70	4.6c, A/pT	35	−2q34-ter,−3p,+3q,−6,−9p,−14q21–23,+15q22–25, −17p,+20
28	C39	34	IIb	60	Neg.	70	4.0c, T	45	−3p,−4p,+5p,−5q33-ter,−13
29	C40	50	IIIa	90	5	30	5.2c, A	16	+3q21-ter,+19q
30	C42	81	IIIb	90	<2	50	6.2c, A	16	−2q,−3p,++3q,−4q,++5p,+5q,−8pter-23,−9p, −10p,−11,−12p,−13, +14q22-ter,+17,+19,+20

[a]MIB-1, DO-1, and WAF-1 immunohistochemical results are presented as percentages of the tumor cells that reacted with the respective antibody. DNA histograms are classified as tetraploid (T), aneuploid/proliferating tetraploid (A/pT), and aneuploid (A) (see also Fig. 1). n.d., not determined. The HPV types are provided as determined by dot blot analysis. Neg., negative for detection of HPV sequences. The CGH column shows the chromosomal aberrations detected in individual cases.

gram, the details of which are described by du Manoir et al. (1995). Average ratio profiles were computed as the mean value of at least 8 ratio images (Fig. 2D,E). We used average ratio profiles to identify chromosomal copy number changes in all cases.
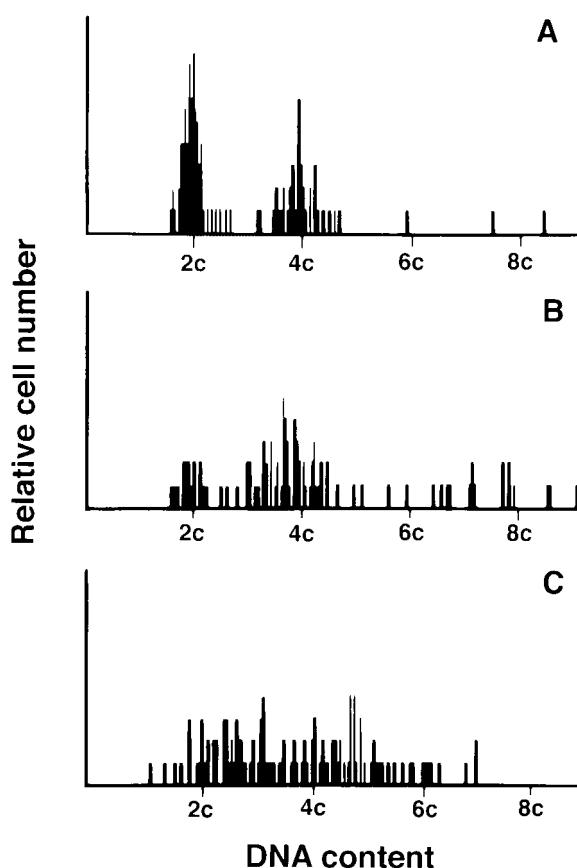
Figure 1.   Examples of tetraploid (**A**), aneuploid/proliferating tetraploid (**B**), and aneuploid (**C**) DNA content histograms. See Materials and Methods for details of the classification.

RESULTS

We recently identified a gain of chromosome arm 3q as a recurrent chromosomal aberration that occurs at the transition from preinvasive to invasive, early-stage cervical carcinomas (Heselmeyer et al., 1996). We performed the present study to address the question whether gain of 3q is maintained when the tumors progress, and to identify additional chromosomal aberrations that might be required for further progression of cervical carcinomas. We now report the mapping of chromosomal gains and losses in 30 cases of advanced-stage cervical carcinomas. The clinical data are summarized in Table 1.

CGH Analysis

Genomic tumor DNA was extracted from 50 μm formalin-fixed, paraffin-embedded tissue sections that were microdissected from representative regions of the tumor (Fig. 2A). An example of the CGH analysis is presented for case 15 as a ratio image of a representative metaphase cell and as an

average ratio profile of 10 metaphases of the same case (Fig. 2D,E). Average ratio profiles were used for mapping of copy number changes in all instances. Chromosomal aberrations were detected in all 30 cases; the average number of chromosomal aberrations per case was 8.2. Despite the high number of chromosomal aberrations, a recurrent and specific pattern emerged in advanced-stage cervical carcinomas: the most frequent copy number alteration was a gain of chromosome arm 3q, which occurred in 23 of the 30 cases (77%). In 4 cases, the gain of 3q was present as a high level copy number increase (amplification). The consensus region of 3q amplification was mapped to chromosomal bands 3q26-27. Gain of genetic material was also identified on chromosome arms 1q (14/30), 5p (9/30), 6p (8/30), and 20p (7/30). Whole-arm amplifications were frequently observed on 5p. Regional high level copy number increases (amplifications) were mapped to chromosomal bands 3q26-27, 9p23-24, 11q22-23, and 12p13. The most common chromosomal copy number decrease was mapped to chromosome arm 3p (15/30), band 2q36-37 (10/30), 6q (8/30), 8p (7/30), 4p (7/30), and 13q (8/30). A summary of chromosomal gains and losses is presented in Figure 3.

HPV Genotyping

The presence of HPV genomes was analyzed by PCR in the same DNA as for CGH. All samples could be analyzed successfully with a primer for the β-globin gene used as a control. HPV genomes were not detected in 5 cases. Twenty-five tumor samples yielded positive test results. Invariably, the detected HPV belonged to the high-risk group. HPV 16 accounted for 48% of the infections, followed by HPV 45 (6/25). The results are summarized in Table 1.

DNA Ploidy Measurement

DNA ploidy was measured on consecutive tissue sections by image cytometry. Image cytometry complements the CGH analyses by providing information of the DNA ploidy that cannot be gained from CGH experiments alone. None of the tumors showed a normal diploid DNA content. Most cases had crude aneuploidy, with either proliferating tetraploid cell populations or highly aneuploid cell populations; more than 20% of the cells exceeded 5c values. The results are presented in Table 1.

Immunocytochemistry

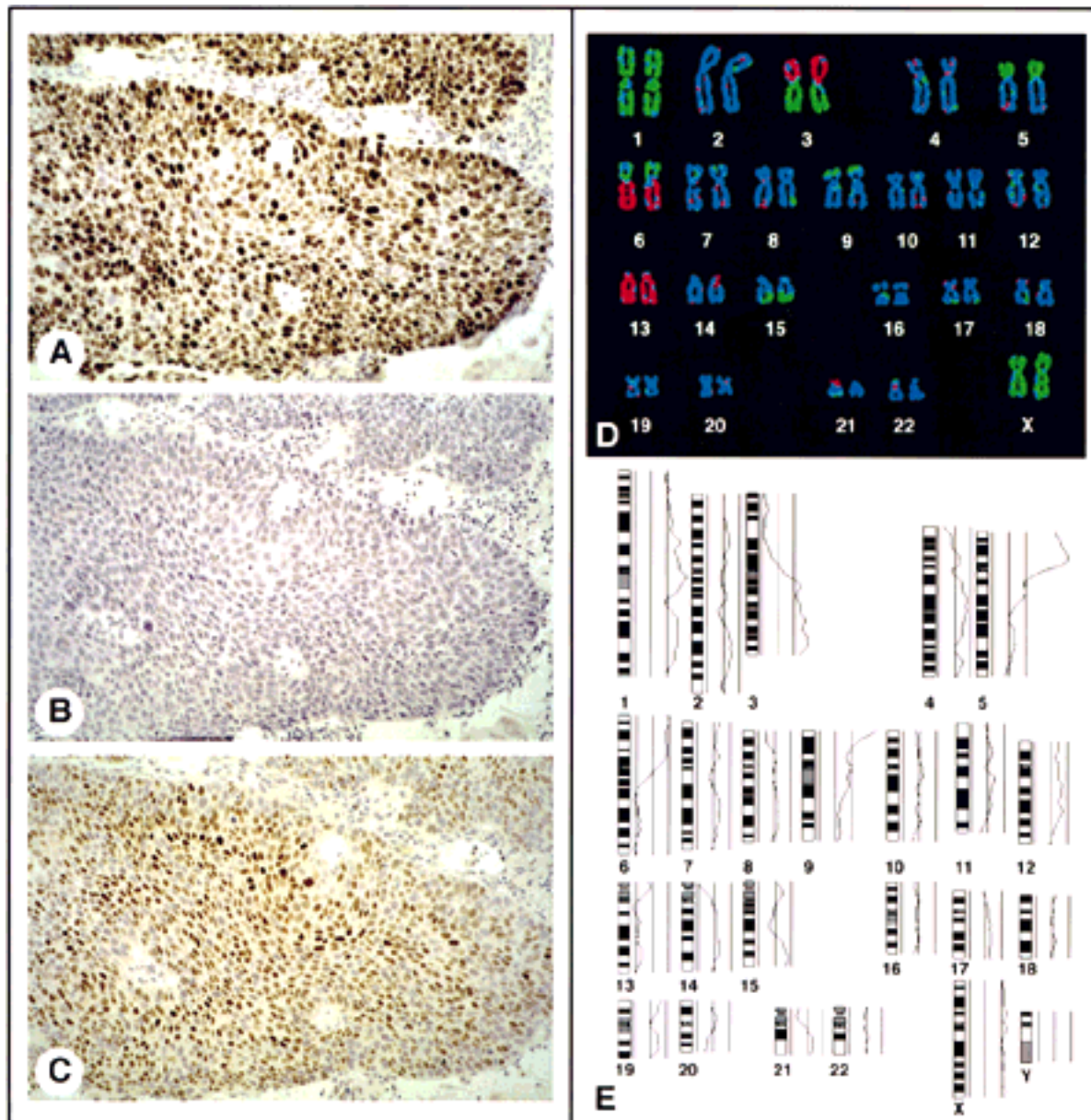The antibodies MIB-1, which is directed against the Ki-67 antigen, DO-1, which reacts with TP53,

Figure 2. Immunohistochemical detection of Ki-67 (**A**), TP53 (**B**), and p21/WAF-1 (**C**) of case 15. Immunoreactive nuclei appear dark brown. The tissue was counterstained with H&E, which appears blue. In this case, the proliferative activity was increased in 80% of the cells (A), TP53 expression was negligibly low (<2%) (B), and p21/WAF-1 immunoreactivity was present in 60% of the cells (C). **D:** A ratio image of case 15. Blue indicates a balance between tumor and test genomes, red reflects a loss of genetic material in the tumor DNA, and green shows regions that are gained in the tumor. **E:** The average ratio profile that was computed on the basis of 10 ratio images. The three vertical lines on the right side of the chromosome ideograms reflect different values of the fluorescence ratio between tumor and normal DNA. The values are 0.75, 1, and 1.25 from left to right, respectively. Copy number changes are present on 1, 3q, 5p, 6p, 9p, and X (gains) and 3p, 6q, and 13 (losses).

and p21/WAF-1, which binds to the p53-activated cell cycle regulator WAF-1, were used for immunohistochemical staining of consecutive tissue sections. Proliferative activity was, in general, markedly increased, with most tumor samples showing strong reactivity in 70–90% of the cells. With the exception of two cases (11 and 20), immunoreactivity with DO-1 (directed against TP53) was negative or only slightly increased. Immunoreactivity for p21/WAF-1 was generally elevated. The strong
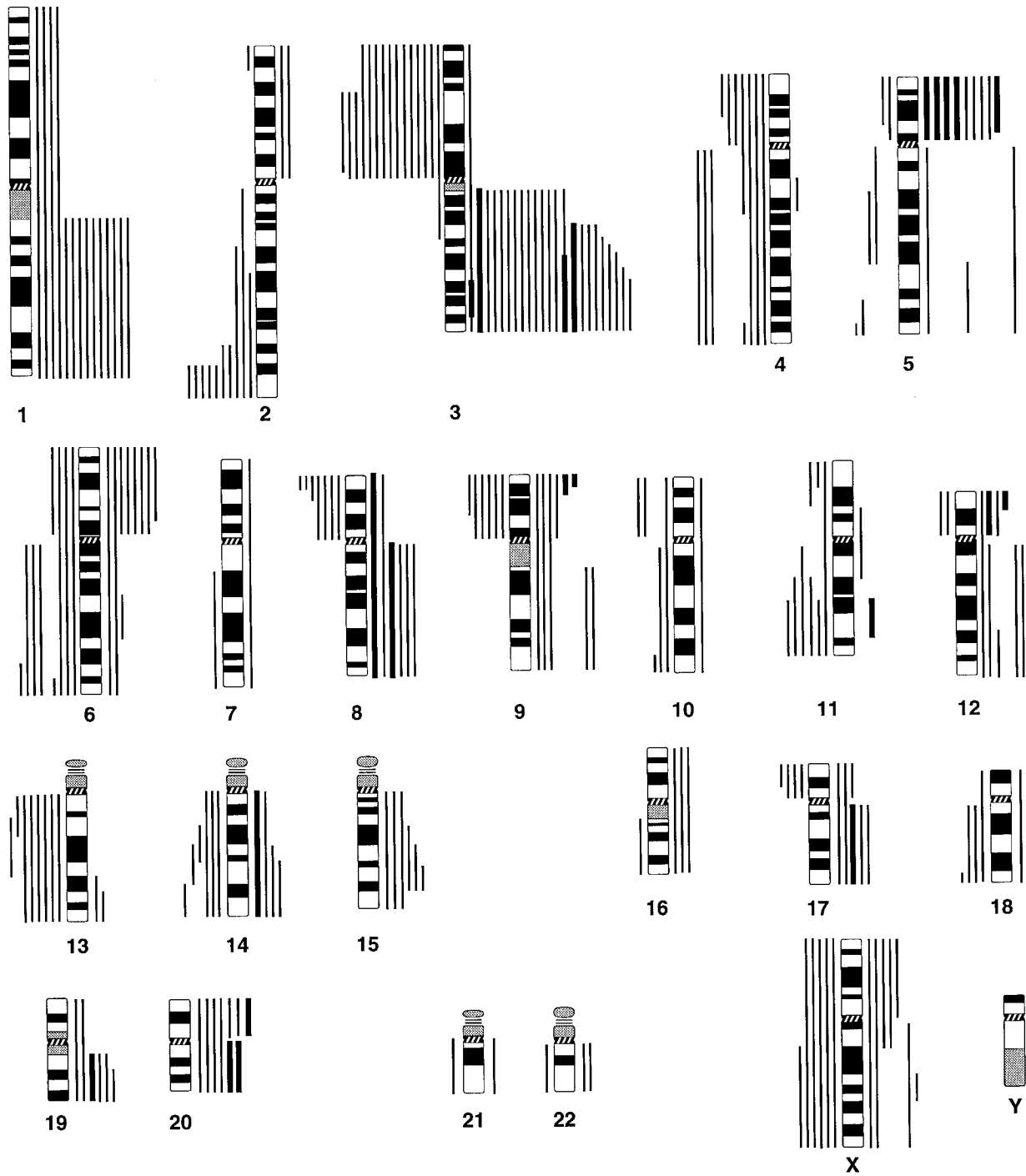
Figure 3. Karyogram of gains and losses in 30 cases of advanced-stage cervical carcinoma. Lines on the right side of the ideograms reflect copy number increases; lines on the left represent copy number decreases. Heavy lines symbolize high level copy number increases (amplifications).

expression of TP53 in cases 11 (60% of the cells reacting) and 20 (80%) did not affect the expression pattern of WAF-1. Examples of the immunohisto-chemical findings (case 15) are provided in Figure 2A–C. The quantification of the data is summarized in Table 1.

DISCUSSION

This study summarizes the results of a combined phenotype/genotype analysis of 30 cases of advanced-stage cervical carcinomas. By using CGH as a molecular cytogenetic screening test for copy number changes, we identified a recurrent and stage-specific pattern of chromosomal aberrations in these tumors. Proliferation analysis and measurement of the cellular DNA content revealed that advanced-stage cervical tumors were highly proliferative and aneuploid, frequently with stemlines in the tetraploid region. We examined the expression status of TP53 and p21/WAF-1 to understand the involvement of p53 in the progression of cervical carcinomas. High-risk HPV types were detected in 83% of the tumors.

CGH analysis uncovered a recurrent pattern of chromosomal aberrations in advanced-stage disease. Most commonly, we observed a gain of chromosome arm 3q (23 of 30 tumors). In 4 cases, these gains were present as high level copy number increases. The smallest amplicon could be mapped to chromosomal bands 3q26-27. It is likely that this region harbors a gene or genes whose gain of function is important in cervical carcinogenesis. Other characteristic copy number increases were mapped to chromosome arm 5p, remarkably often as high level copy number increases, and to chromosome arm 1q. The involvement of chromosome arm 1q was suspected by G-banding analysis (Popescu and DiPaolo, 1992). Atkin et al. (1990) have observed a marker chromosome in many cervical carcinomas and suggested that it reflects an isochromosome 4p or 5p. The frequent gain of 5p material in our collection of advanced cervical carcinomas suggests that this marker is likely to be derived from an i(5)(p10). It is noteworthy in this context that Mitra and colleagues (1995) identified loss of heterozygosity (LOH) on chromosome arm 5p in preinvasive lesions. However, the gain of 5p seems to be a recurrent event during progression to advanced-stage carcinomas. Consistent losses, indicative of chromosomes where tumor suppressor genes reside, were mapped to chromosome arms 3p, 8p, 13q, and 2q. The loss of chromosome arm 3p is also a frequent finding in LOH studies (Mullokandov et al., 1996). Chromosome arm 11q showed copy number decreases in 6 cases. LOH studies with markers for this chromosome arm indicated a recurrent loss in cervical carcinomas (Srivatsan et al., 1991). Twenty-two whole-arm or band-specific amplifications were mapped. Compared to the number of amplifications in small cell lung carcinomas (Ried et al., 1994; Levin et al., 1995), aneuploid breast carcinomas (Kallioniemi et al., 1994; Ried et al., 1995), and glioblastomas (Schröck et al., 1994; Kim et al., 1995), this number is low. The average number of chromosomal aberrations (ANCA) per case amounts to 8.2. This is similar to the ANCA index established by use of CGH analysis of glioblastomas, where it is 8.3 (Schröck et al., 1994), and aneuploid breast carcinomas, where it is 6.8 (Ried et al., 1995), indicating a high genetic instability.

We have previously analyzed, by CGH, DNA copy number changes that occur in the progression from normal cervical epithelium to stage I invasive carcinomas (Heselmeyer et al., 1996). Nine of 10 of the invasive carcinomas exhibited a gain of chromosome arm 3q, whereas this aberration was present in only 1 of 13 severe dysplasias. It is, therefore, obvious that the gain of chromosome arm 3q occurs at the transition from preinvasive to invasive disease. The analysis of 30 cases of advanced-stage carcinomas shows that this particular chromosomal aberration was maintained in the majority of the tumors (77%). However, additional chromosomal changes emerged that include gains of chromosome arms 1q and 5p, as well as losses of 2q. The analyses allow us to identify a stage-specific pattern of chromosomal aberrations, in which 3q gain is an early event that coincides with the progression from preinvasive lesions to stage I invasive carcinomas. The gains of chromosome arms 1q and 5p, however, occur in advanced stages of tumor progression.

The immunohistochemical pattern in advanced cervical carcinomas can be summarized as follows: TP53 expression is relatively low, and only two cases revealed increased immunoreactivity. p21/WAF-1 is generally elevated. This is in sharp contrast to colon carcinomas, where TP53 levels are dramatically increased and immunoreactivity for p21/WAF-1 is low (Ried et al., 1996). We hypothesized that the difference in immunophenotype is due to the frequent presence of high-risk HPV in advanced-stage cervical carcinomas. Interestingly, a strikingly similar immunophenotypic response was observed in a series of squamous cell carcinomas of the anal canal, which, like cervical carcinomas, are often associated with HPV infection. Apparently the presence of HPV is not sufficient to inactivate all p53 activity, because p21/WAF-1 is still increased (Heselmeyer et al., in press).

The correlation of phenotype/genotype in cervical carcinomas can be summarized as follows: HPV infection is present in mildly dysplastic lesions and persists throughout advanced stages. Virtually all HPVs in lesions with increased levels of dysplasia belong to the high-risk group. Proliferative activity reaches maximum levels in severely dysplastic lesions and remains elevated during the progression to advanced-stage tumors. The tetraploidization that we observed already in mild dysplasia is replaced by cells with profound aneuploidy (Heselmeyer et al., 1996). The gain of chromosome arm 3q is an early event in cervical carcinogenesis and occurs at the transition from premalignant lesions to invasive carcinoma, stage I. During progression to advanced-stage disease, additional chromosomal aberrations are acquired, namely, the gain of chromosome arms 1q and 5p, as well as the loss of chromosomal bands 2q36-37. The gain of chromosome 3q, however, persists.

ACKNOWLEDGMENTS

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